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(54) **Benzodiazepines assay, tracers, immunogens and antibodies.**

(57) The present invention is directed to a fluorescence polarization assay for benzodiazepines and their metabolites, to the various components needed for preparing and carrying out such an assay and to methods of making these components. Specifically, tracers, immunogens and antibodies are disclosed, as well as methods for making them. The assay is conducted by measuring the degree of polarization retention of the fluorescence resulting when a sample mixed with antiserum and tracer is irradiated with plane polarized light.

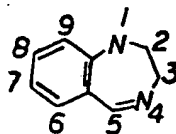


FIG. 1

BENZODIAZEPINES ASSAY, TRACERS, IMMUNOGENS AND ANTIBODIES1. Technical Field

The present invention relates to a method and reagents for a fluorescence polarization immunoassay procedure for determining the presence or amount of benzodiazepines in fluids, especially biological fluids such as urine, serum or plasma, and to a method of making the reagents. More specifically, the invention relates to (1) reagents (tracers and antibodies) for determining the presence or amount of benzodiazepines and/or their metabolites in a sample; (2) immunogen compounds used to raise the antibodies; (3) synthetic methods (for making the tracer and immunogen compounds); and (4) analytical methods for conducting the assay.

2. Background Art

Benzodiazepine drugs constitute a class of compounds, most of which contain the benzodiazepine ring structure (Figure 1) bearing an aromatic substituent at the 5-position. They are used clinically as sedatives, hypnotics, muscle relaxants, anxiolytics, anti-epileptics and in the treatment of alcohol abuse. One benzodiazepine, diazepam, can cause a slight euphoric state and is also used illicitly either alone or as an adulterant in other illicit compounds.

Benzodiazepines are extensively metabolized, primarily by dealkylation at the 3-position in the case of 7-chloro-benzodiazepines. The 7-nitro-benzodiazepines metabolize to 7-amino- and 7-acetamido forms as well. The hydroxylated metabolites are excreted primarily as glucuronide conjugates.

Few fatalities have been attributed to benzodiazepine ingestion alone. Benzodiazepines are generally considered to be safe compounds and this attitude has promoted their use. They are among the most frequently prescribed drugs in the Western world. The widespread use of benzodiazepines has led to accidental overdosage as well as intentional abuse, and analysis for their presence is typical during abused drug testing and in emergency rooms. Rapid, reliable, selective and accurate detection methods for benzodiazepines and their metabolites aid in this endeavor.

The biological fluid most frequently tested is urine. Urine samples are more accessible than blood samples, and other biological fluids have not been extensively investigated for use in assays.

In the past, the presence of benzodiazepines in urine has typically been detected by thin layer chromatography (TLC) or enzyme immunoassay (EIA) with confirmation of levels in serum or plasma by high performance chromatography (HPLC) or gas chromatography (GC). These methods for analysis in urine are not without drawbacks. The TLC methods require sample extraction procedures and assay time is lengthy. EIAs involve enzyme reactions and have the following disadvantages: 1) the reagents are relatively unstable; 2) any components in the biological samples which may affect absorbance readings of the products of the enzyme reaction in an EIA (such as enzyme inhibitors or enzymes which catalyze similar reactions) will affect the assay results; and 3) EIAs measure absorbance, and any compounds in the biological samples (such as bilirubin or other chromophores) will affect the accuracy of the results obtained from these assays.

In assays for drugs and other substances, fluorescence polarization competitive binding immunoassays have provided a more satisfactory alternative. Typically, competitive binding immunoassays are used for measuring ligands in a test sample. (For the purposes of this disclosure, a "ligand" is a substance of biological interest to be determined quantitatively by a competitive binding immunoassay technique).

Benzodiazepine antigen conjugates and antibodies have been described in U.S. Patent No. 4,243,654 to Schneider et al., in U.S. Patent No. 4,083,948 to Davis et al., in U.S. Patent No. 4,191,738 to Dixon, and in U.S. Patent No. 4,046,636 to Ullman et al.

The present invention offers an advance in the art beyond that described supra, particularly in that effective immunogens, selective antibodies, highly sensitive fluorescent tracers, methods for preparing the antibodies and fluorescent tracers, and an assay employing the tracers and antibodies are provided specifically for the determination of one or more benzodiazepines or benzodiazepine metabolites in a sample. An assay conducted in accordance with the present invention is particularly accurate, as will be explained infra.

SUMMARY OF THE INVENTION

The present invention is directed to haptens and immunoassays used to raise antibodies which can be employed in assays for benzodiazepines and benzodiazepine metabolites; to tracers for use in fluorescence polarization immunoassays for benzodiazepines and their metabolites; to methods for synthesizing the haptens, immunogens and tracers; and to methods for conducting such assays.

A first aspect of the invention relates to the discovery of unique haptens and immunogens having novel structures. According to this first aspect of the invention, the haptens and immunogens can both be represented by the structural formula shown in Figure 2, wherein:

- 10 X is CH, N or C-halogen;
R₁ is -H, -CH₃ or -R-Z-Q;
R₂ is -H or -OH;
R₃ is -O or a nonbonding electron pair;
R₄ is -R-Z-Q when R₁ is -H or -CH₃, or halogen, -NO₂, -NH₂ or -NHCOCH₃ when R₁ is -R-Z-Q;
- 15 R is a linking group consisting of from 0 to 20 carbon atoms and heteroatoms, including not more than twelve heteroatoms, arranged in a straight or branched chain and containing up to two ring structures, with the proviso that not more than four heteroatoms may be linked in sequence, nor may more than two sulfur or nitrogen or one oxygen atom be linked in sequence;
Z is C=O, C=NH, NH, NCH₃, N=N, SO₂ or CH₂; and
- 20 Q is hydrogen, hydroxyl or a leaving group when the compounds are employed as haptens. (For purposes of this disclosure, a "leaving group" is a halogen, an acyloxy group [including carbonate ester], a N-succinimidyloxy or N-phthalimidyloxy group, an alkoxy or phenoxy or substituted phenoxy group, an N-imidazolyl group, a 1-benzotriazolyl group or any of the other similar activating groups well known to those skilled in the art). Q is a poly(amino acid), a poly(amino acid) derivative or another immunogenic
- 25 carrier substance when the compounds are employed as immunogens.

A second aspect of the invention relates to methods for preparing immunogens from the novel haptens. According to this aspect of the invention, methods are provided for making immunogens by chemically combining any compound represented by the structural formula shown in Figure 2 wherein:

- X is CH, N or C-halogen;
- 30 R₁ is -H, -CH₃ or -R-Z-Q;
R₂ is -H or -OH;
R₃ is -O or a nonbonding electron pair;
R₄ is -R-Z-Q when R₁ is -H or -CH₃, or halogen, -NO₂, -NH₂ or -NHCOCH₃ when R₁ is -R-Z-Q;
- R is a linking group consisting of from 0 to 20 carbon atoms and heteroatoms, including not more than
- 35 twelve heteroatoms, arranged in a straight or branched chain and containing up to two ring structures, with the proviso that not more than four heteroatoms may be linked in sequence, nor may more than two sulfur or nitrogen or one oxygen atom be linked in sequence;
Z is C=O, C=NH, SO₂, NH, NCH₃ or CH₂; and
Q is hydrogen, hydroxyl or a leaving group (with the proviso that when Z is CH₂, Q may not be
- 40 hydrogen);
with a macromolecular or particulate carrier substance such as a poly(amino acid), a poly(amino acid) derivative or other macromolecular carrier or a synthetic polymeric bead bearing reactive functional groups on its surface.

A third aspect of the invention relates to antibodies raised by the novel immunogens. According to this aspect of the invention, antibodies are prepared in response to a compound or substance synthesized as described, supra, employing in vivo or in vitro techniques, and methods well known to those skilled in the art.

A fourth aspect of the invention relates to the discovery of unique tracers having novel structures and to compounds which serve as synthetic precursors for them. According to this aspect of the invention, these tracers and tracer precursors can be represented by the general structural formula shown in Figure 2 of the drawings, wherein:

- X is CH, N or C-halogen;
R₁ is -H, -CH₃ or -R-Z-Q;
R₂ is -R-Z-Q when neither R₁ nor R₄ is -R-Z-Q or it is -H or -OH;
- 55 R₃ is -O or a nonbonding electron pair;
R₄ is -R-Z-Q when neither R₁ nor R₂ is -R-Z-Q, or it is -halogen, -NO₂, -NH₂ or -NHCOCH₃;
- R is a linking group consisting of from 0 to 20 carbon atoms and heteroatoms, including not more than twelve heteroatoms, arranged in a straight or branched chain and containing up to two ring structures, with

the proviso that not more than four heteroatoms may be linked in sequence, nor may more than two sulfur or nitrogen or one oxygen atom be linked in sequence;

Z is NH, CO, SO₂ or C=NH; and

Q is -H, -OH, a leaving group or fluorescein or a derivative of fluorescein.

- 5 When Q is fluorescein or a derivative of fluorescein, the compound can be used as a tracer; when Q is -H, -OH or a leaving group, the compound can be used as a precursor to a tracer.

A fifth aspect of the invention relates to methods for preparation of the novel tracers. According to this aspect of the inventions, a tracer is made by chemically coupling a compound represented by the general structural formula shown in Figure 2 of the drawings wherein:

- 10 X is CH, N or C-halogen;

R₁ is -H, -CH₃ or -R-Z-Q;

R₂ is -R-Z-Q when neither R₁ nor R₄ is -R-Z-Q or it is -H or -OH;

R₃ is -O or a nonbonding electron pair;

R₄ is -R-Z-Q when neither R₁ nor R₂ is -R-Z-Q, or it is halogen, -NO₂, -NH₂ or -NHCOCH₃;

- 15 R is a linking group consisting of from 0 to 20 carbon atoms and heteroatoms, including not more than twelve heteroatoms, arranged in a straight or branched chain and containing up to two ring structures, with the proviso that not more than four heteroatoms may be linked in sequence, nor may more than two sulfur or nitrogen or one oxygen atom be linked in sequence;

Z is NH, CO, SO₂ or C=NH; and

- 20 Q is -H, -OH, a leaving group with fluorescein or a derivative of fluorescein.

A sixth aspect of the invention relates to an analytical method, i.e., a method for conducting an assay employing as reagents the tracers and antisera to benzodiazepines and benzodiazepine metabolites described *supra*. According to this sixth aspect of the invention, an improved fluorescence polarization immunoassay is provided, which can be performed by contacting a fluid containing, or suspected of
25 containing benzodiazepine(s) or metabolite(s) therefrom with antisera to benzodiazepine(s) and a fluorescein-labelled benzodiazepine derivative capable of producing a detectable fluorescence polarization response to the presence of the antiserum in a homogeneous solution, while passing plane-polarized light through the homogeneous solution, and measuring the fluorescence polarization response therefrom.

- 30 A seventh aspect of the invention relates to the elimination of potential fluorescence interferences by riboflavin or other fluorescent chromophores. Sodium iodide is added either directly to each sample or to one or more of the reagents utilized in the assay, wherein it quenches the fluorescence present, thus eliminating fluorescence interference.

Further objects and attendant advantages of the invention will be best understood from a reading of the following detailed description taken together with the Figures and the Examples.

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BRIEF DESCRIPTION OF THE DRAWINGS

- In the following Figures the symbol "FI" represents fluorescein or a fluorescein derivative and the
40 various other symbols are noted in the Detailed Description.

Figure 1 shows the basic structure of the class of benzodiazepines to be quantitatively or qualitatively determined in accordance with the present invention, including the numbering scheme used for these compounds.

- 45 Figure 2 shows a general structural formula for the haptens, immunogens, tracers and tracer precursors of the present invention.

Figure 3 shows the alternate structural formulae together with names and numbering schemes for the fluorescein moiety included in the tracers of the present invention.

Figure 4 shows a general structural formula for the preferred immunogens of the present invention.

Figure 5 shows a structural formula for the most preferred immunogen of the present invention.

- 50 Figure 6 shows a general structural formula for the preferred tracers of the present invention.

Figure 7 shows the various linkages that couple the fluorescein moiety to the tracer precursors of Figure 2.

Figures 8 through 12 show various examples of hapten reactants which can be used to form the immunogens and tracers of the present invention.

- 55 Figure 13 shows an example of an immunogen prepared in accordance with the present invention.

Figures 14 through 20 show various examples of structures of tracers in accordance with the present invention.

Figures 21 through 24 show various examples of synthetic intermediates employed in the synthesis of hapten and tracer precursors of the present invention.

5 DETAILED DESCRIPTION OF THE INVENTION

The various aspects of the invention will now be discussed in detail in relation to the Figures.

The present invention involves the use of fluorescein and derivatives of fluorescein. In particular, a necessary property of fluorescein and its derivatives for the usefulness of the tracer compounds described
 10 herein is the fluorescence of fluorescein. Fluorescein exists in two tautomeric forms, illustrated in Figure 3, depending on the acid concentration (pH) of the environment. In the open (acid) form, there are a number of conjugated double bonds which make that form of fluorescein (and compounds containing a fluorescein moiety) capable of absorbing blue light and emitting green fluorescence after an excited state lifetime of about four nanoseconds. When the open and closed forms coexist, the relative concentration of molecules
 15 in the open and closed forms is easily altered by adjustment of the pH level. Generally, the tracer compounds of the present invention exist in solution as biologically acceptable salts such as sodium, potassium, ammonium and the like, which allows the compounds to exist in the open fluorescent form, when employed in the analytical methods of the present invention. The specific salt present will depend on the buffer employed to adjust the pH level. For example, in the presence of a sodium phosphate buffer, the
 20 compounds of the present invention will generally exist in the open form, as a sodium salt.

As used herein, the term "fluorescein," either as an individual compound or as a component of a larger compound, is meant to include both the open and closed forms, if they exist for a particular molecule, except in the context of fluorescence. An open form is necessary for the fluorescence to occur.

The numbering of carbon atoms of the fluorescein molecule varies, depending upon whether the open
 25 or closed form of the molecule is considered. Accordingly, the literature concerning fluorescein and its compounds is not uniform as to carbon atom numbering. In the closed form, the para carbon to the carbonyl of the lactone on the phenyl ring is numbered "6." In the open form, the para carbon to the carboxylic acid group on the phenyl ring is numbered "5" (see Figure 3). In this disclosure the numbering of the closed form is adopted because the raw materials used in the syntheses are most popularly
 30 numbered with that system. The carbon atom of fluorescein and its compounds which is opposite the carboxyl group is therefore numbered "6" for the purposes of the present disclosure.

A tracer in solution which is not complexed to an antibody is free to rotate in less than the time required for absorption and re-emission of fluorescent light. As a result, the re-emitted light is relatively randomly oriented so that the fluorescence polarization of a tracer not complexed to an antibody is low, approaching
 35 zero. Upon complexing with a specific antibody, the tracer-antibody complex thus formed assumes the rotation of the antibody molecule, which is slower than that of the relatively small tracer molecule, thereby increasing the polarization observed. Therefore, when a ligand competes with the tracer for antibody sites, the observed polarization of fluorescence of the resulting mixture of the free tracer and tracer antibody complex assumes a value intermediate between that of the tracer and that of the tracer-antibody complex. If
 40 a sample contains a high concentration of the ligand, the observed polarization value is closer to that of the free ligand, i.e., low. If the test sample contains a low concentration of the ligand, the polarization value is closer to that of the bound ligand, i.e., high. By sequentially exciting the reaction mixture of an immunoassay with vertically and then horizontally polarized light and analyzing only the vertically polarized component of the emitted light, the polarization of fluorescence in the reaction mixture may be accurately
 45 determined. The precise relationship between polarization and concentration of the ligand to be determined is established by measuring the polarization values of calibrators with known concentrations. The concentration of the ligand can be interpolated from a standard curve prepared in this manner.

The particular antibodies and tracers formed in accordance with this invention have been found to produce very good assays, as discussed infra.

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1. The Reagents

Both the immunogens and the tracers of the present invention can be represented by the general structural formula set forth in the Summary of the Invention, and illustrated in Figure 2.

- 5 The objective is to have competition between benzodiazepines and the benzodiazepine metabolites and the tracer for the recognition sites of the antibody. Great variations in the structure of the haptens and tracers are allowed in achieving this goal. For the purpose of this invention, "haptens" are precursors of the immunogens, comprising generally a substituted benzodiazepine derivative bearing a group suitable for linking to an immunologically active carrier.

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a. The Structure of the Immunogens

- Usable antibodies can be produced from a variety of benzodiazepine derivatives. Immunogens made from compounds functionalized either at the 1-or 7-position on the ring system can produce antibodies in animals; such antibodies are useful in benzodiazepines and benzodiazepine metabolites assay according to this invention when combined with the appropriate tracer.

- The immunogens of the present invention have the general structural formula shown in Figure 2 and, in a preferred form of the invention, the immunogens are derived from the general structural formula shown in Figure 4. The immunogens can be prepared by coupling a compound of the class shown in Figure 2 with a poly(amino acid) or a derivative of a poly(amino acid) or other immunologically active carrier as will be discussed in the context of the synthetic method and the Examples below.

- Although substitution at either the 1-or 7-position is equally preferred from a structural point of view, starting materials are more readily available and the synthesis is greatly simplified for the 7-position derivatives. However, the probability of obtaining useful antisera from a given animal appears to be greater for derivatives of the 1-position. Accordingly, the 1-position derivatives represented by the general structural formula of Figure 4 (wherein R, R₂ and R₃ are defined as supra, Z is C=O, C=NH, SO₂, NH, NCH₃ or CH₂ and Q is an immunogenic carrier) are the preferred embodiment of this aspect of the invention. In the most preferred form of this aspect of the invention, the immunogens are represented by the structural formula of Figure 5. This structure is preferred because it provides antisera with sensitivity to a broad range of benzodiazepines and benzodiazepine metabolites while excluding other drugs and endogenous substances. Although bovine serum albumin is the poly(amino acid) in this most preferred form, it should be understood that various protein carriers can be employed, including albumins, serum proteins, e.g., globulins, ocular lens proteins, lipoproteins and the like. Illustrative protein carriers include bovine serum albumin, keyhole limpet hemocyanin, egg ovalbumin, bovine gamma globulin, thyroxine binding globulin, etc. Alternatively, synthetic poly(amino acids) having a sufficient number of available amino groups such as those on lysine or ornithine residues can be employed, as can many other synthetic or natural polymeric materials bearing reactive functional groups. In addition, carbohydrates, yeasts, polysaccharides or any other substance that can be used as an immunological carrier can be conjugated to the hapten to produce an immunogen.

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b. The Structure of the Tracers

- The possible variations in the structure of the tracers of the present invention are even greater than the possible variations in the structure of the haptens. The tracers have the general structural formula shown in Figure 2. In a preferred form of the invention, the tracers have the structural formula shown in Figure 6, wherein R is as defined supra and Z is C=O, NH, SO₂ or C=NH.

- The tracer is a benzodiazepine derivative that is linked to a fluorescein derivative by, for example, an amido, amidino, triazinylamino, carbamido, thiocarbamido, carbamoyl, thiocarbamoyl, or sulfonylcarbamoyl group, as shown in Figure 7. The tracers are prepared by linking the appropriate fluorescein derivative to a benzodiazepine derivative containing an amino, carboxylic acid, sulfonic acid, mercapto, hydroxy, imidate, hydrazide, isocyanate, thioisocyanate, chloroformate, chlorothioformate, chlorosulfonylcarbamoyl, or similar group, as will be discussed in the context of the synthetic method and the Examples below.

- By way of example, any of the following fluorescein derivatives can be used as reactants:

- 55 FI-NH₂ fluorescein amine
FI-CO₂H carboxyfluorescein
FI-NHCOCH₂I iodacetamidofluorescein

FI-NHCOCH₂Br -bromoacetamidofluorescein 2,4-dichloro-1,3,5-triazin-2-ylaminofluorescein (DTAF) 4-chloro-6-methoxy 1,3,5-triazin-2-ylaminofluorescein
 FI-NCS fluorescein thioisocyanate
 FI-CH₂NH₂ 11-aminomethyl fluorescein

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2. The Antibodies

The antibodies of the present invention are prepared by eliciting a response in sheep or rabbits to the immunogens described supra. The immunogen is administered to animals or to in vitro cultures of immunocompetent cells by a series of inoculations, in a manner well known to those skilled in the art.

3. Synthetic Methods

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Both the immunogens and the tracers of the present invention can be made from a precursor having the general structural formula shown in Figure 2.

20 a. The Synthesis of the Immunogens

The immunogens of the present invention are made by coupling a hapten, such as that shown by the general structure of Figure 2, to a poly(amino acid). The poly(amino acid) moiety can be linked to the hapten by an amide, an amidine, an alkyl, a urea, a thiourea, a carbamate, or a thiocarbamate linkage. In a preferred embodiment, the poly(amino acid) is bovine serum albumin (BSA) and the hapten is shown in Figure 8. The hapten is preferably coupled under conditions normally used to form amide linkages, which conditions are well known to those skilled in the art. The immunogens are prepared by coupling a hapten that contains an -NH₂, -CO₂H, -CONHNH₂, -CNOR, -CHO, -Br, -I, -NCO, -NCS, -OCOCI, -SO₂Cl or -OCSCI group to a poly(amino acid). Haptens containing an NH₂ group can be coupled by activating the carboxylic acid group on the poly(amino acid) in the presence of the -NH₂ group. For aromatic amines, the diazonium salt method can be used. The diazonium salt, prepared by mixing the amine with sodium nitrite in acid solution, is added to the poly(amino acid). Activation of the carboxylic acid groups on the poly(amino acid) can be accomplished by mixing the hapten and the poly(amino acid) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N,N'-dicyclohexylcarbodiimide (DCC), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methoxy-p-toluenesulfonate, or the like. Carboxylic acid-containing haptens are also coupled by the in situ activation method (EDC) or the active ester method, as described below in the tracer synthesis section. For -CONHNH₂, coupling is performed as for a non aromatic amino group. A -CNOR compound, which is prepared from the corresponding cyano compound, is coupled directly to the poly(amino acid). A -CHO compound is coupled to the poly(amino acid) by reductive amination. The poly(amino acid) is mixed with the -CHO hapten and the resulting imine is reduced with a borohydride reducing agent such as sodium cyanoborohydride to yield alkylated amines on the poly(amino acid). Isocyanate (-NCO) and isothiocyanate (-NSC) compounds, which are prepared from the corresponding amino compound, and chloroformate (-OCOCI) and chlorothioformate (-OCSCI) compounds, which are prepared from the corresponding alcohol compound, produce urea, thiourea, carbamate and thiocarbamate linkages, respectively. This is accomplished by directly coupling the hapten to the poly(amino acid).

The syntheses of the above haptens (immunogen precursors) are accomplished in very similar ways. Figure 4 shows an immunogen precursor class in accordance with a preferred embodiment of the method of the present invention.

In general, the 1-substituted haptens are prepared by alkylation of the amide nitrogen of nordiazepam or its derivatives with an ester of an omega-halocarboxylic acid, an omega-haloazide or a sulfonate ester of an omega-azido primary alcohol. The latent or protected functionality is then revealed and the compound is coupled to the poly(amino acid) or other carrier. For example, in the case of the t-butyl ester protected side chain (Figure 21), the protecting group was removed by treating with trifluoroacetic acid, while in the case of the azide-terminated chain, the latent amine functionality was revealed by selective reduction with propane dithiol or triphenylphosphine. It is possible to alkylate nordiazepam or its derivatives with other bifunctional alkylating agents such as bromoacetonitrile, bromoacetaldehyde dimethylacetal and the like.

Aldehydes or ketones can be derivatized by known methods to a variety of compounds containing a suitable group useful for coupling to a carrier protein, such as, Wittig reaction, condensation with hydrazine compounds, reductive amination with amino compounds or the like.

Aldehydes and ketones can also be condensed with (aminohydroxy)alkylcarboxylic acids, such as $\text{NH}_2\text{OCH}_2\text{CO}_2\text{H}$, to produce substituted oxime derivatives. The oxime alkyl carboxylic acid derivatives can be partially reduced to the corresponding (aminohydroxy)alkylcarboxylic acid derivatives. The same type of condensation and reduction can be accomplished with hydrazine and hydrazine derivatives.

Nitrile derivatives can be converted to alkoxy imidates by treating the nitrile with anhydrous alcohol and hydrogen chloride gas. The hydrazide derivatives can be prepared from the corresponding carboxylic acid derivatives by active ester coupling with hydrazine or by reacting hydrazine with the corresponding carboxylic ester derivative. Amines are convertible to the isocyanate or thioisocyanate derivatives and alcohols are convertible to chloroformate and chlorothioformate derivatives by reaction of the amine or the alcohol with phosgene or thiophosgene.

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b. The Synthesis of the Tracers

The tracers of the present invention are made by coupling a fluorescein moiety, or a derivative of fluorescein, to the general structure shown in Figure 2. The fluorescein moiety can be linked to the amino, carboxyl, imidate or alkoxy functional group by an amide, an amidine, a urea, a thiourea, a carbamate, a thiocarbamate, triazinylamino or sulfonylcarbamate linkage, as shown in Figure 7. In the presently preferred embodiment, the fluorescein derivative is 6-(4,6-dichloro-1,3,5-triazin-2-yl) amino)fluorescein, and this is coupled to precursors shown in Figures 9 and 10.

All benzodiazepine derivatives that have a terminal amino group, such as amino, hydrazinyl, hydrazido or the like, are coupled to carboxyfluorescein by the active ester method or the mixed anhydride method, and coupled to fluorescein isothiocyanate, DTAF or alkoxy DTAF by simply mixing the two materials in solution. The amino group can be converted to the isocyanate and thioisocyanate groups by reaction with phosgene and thiophosgene, respectively. These are then condensed with aminofluorescein to produce the tracer.

All benzodiazepine derivatives that have a terminal carboxylic acid group, such as carboxylic acid, (aminohydroxy)alkylcarboxylic acid or the like, are coupled to aminofluorescein and aminomethylfluorescein by the active ester method.

Any benzodiazepine derivatives having a terminal hydroxy group can be coupled to fluorescein by reaction with DTAF, -bromoacetamidofluorescein or fluorescein isothiocyanate in solution. The hydroxy group can be converted to the chlorosulfonylcarbamoyl, chloroformate or chlorothioformate groups by reaction with chlorosulfonylisocyanate, phosgene or thiophosgene, respectively. These derivatives are then coupled to aminofluorescein in solution to produce the tracers.

Benzodiazepine derivatives having a terminal mercapto group are prepared from halides or sulfonate esters and alkali metal sulfides as described supra for azides. They are coupled in solution with -bromoacetamidofluorescein or -bromoacetamidofluorescein.

Benzodiazepine derivatives that have a terminal nitrile group can be prepared from halides or sulfonate esters as described, supra, for azides. They are converted to imidates in anhydrous alcohol in the presence of hydrogen chloride gas. The imidate is then coupled to fluorescein amine in solution to prepare the tracer.

Preparation of the various amino, hydroxy and mercapto derivatives of the benzodiazepine derivatives has been described above in the immunogen preparation sections.

3. The Assay

The particular tracers and antibodies of the present invention have been found to produce surprisingly good results in fluorescence polarization assays for benzodiazepines and benzodiazepine metabolites that can be qualitatively or quantitatively determined in accordance with the present invention. The assay of the present invention provides a more rapid benzodiazepines and benzodiazepine metabolites assay method than most prior art methods, because it requires no specimen treatment before analysis. The assay system accurately detects the presence of benzodiazepines and benzodiazepine metabolites in a sample.

In accordance with the analytical methods of the present invention, i. e., the methods of determining benzodiazepines and benzodiazepine metabolites by a fluorescence polarization immunoassay procedure using the tracer compounds and immunogens of the invention, a sample containing or suspected of containing benzodiazepines or benzodiazepine metabolites is intermixed with a biologically acceptable salt of a tracer and an antibody specific to benzodiazepines and benzodiazepine metabolites and the tracer. The antibody is produced using the immunogen described above. The benzodiazepines and benzodiazepine metabolites and tracer compete for limited antibody sites, resulting in the formation of complexes. By maintaining constant the concentration of tracer and antibody, the ratio of benzodiazepines and benzodiazepine metabolites-antibody complex to tracer antibody complex that is formed is directly proportional to the amount of benzodiazepines and benzodiazepine metabolites in the sample. Therefore, upon exciting the mixture with linearly polarized light and measuring the polarization of the fluorescence emitted by a tracer and a tracer-antibody complex, one is able to determine qualitatively whether or not benzodiazepines and benzodiazepine metabolites are present in the sample.

The results can be quantified in terms of net polarization units and span (in millipolarization units). The measurement of millipolarization units indicates the maximum polarization when a maximum amount of the tracer is bound to the antibody, in the absence of any benzodiazepines and benzodiazepine metabolites. The higher the net millipolarization units, the better the binding of the tracer(s) to the antibody. The span is an indication of the difference between the net millipolarization and the amount of tracer bound to the antibody at the minimum concentration above which the sample is defined as containing benzodiazepines and benzodiazepine metabolites. A larger span provides for a better numerical analysis of data. The preferred antibody-tracer(s) combination has a span of at least 30 millipolarization units, but a span of at least 5 millipolarization units at this minimum detectable concentration is acceptable.

Table I shows the results obtained with various embodiments of the present invention, in terms of span and net millipolarization units. In all instances, bovine serum albumin was used as the protein carrier. As seen from the data in Table I, an assay produced from an immunogen made from the hapten of Figure 5 used in combination with tracers of Figures 17 and 19 provides excellent results. Accordingly, this combination is the most preferred form of the invention. In addition the hapten/tracer(s) combination of Fig. 15/Fig. 16, Fig. 15/Fig. 17, and Fig. 15/Fig. 19 also produced acceptable results and are alternative preferred combinations.

TABLE I

5	Hapten used in Immunogen for Antibody	Tracer(s)	N t Polarization*	Span**
	Fig. 5	Fig. 15	187	35
10	"	Fig. 16	195	80
	"	Mixture of Fig. 15/ Fig. 16	211	35
15	"	Fig. 17	203	71
	"	Fig. 18	157	63
20	"	Mixture of Fig. 15/ Fig. 17	214	43
	"	Fig. 19	251	122
25	"	Mixture of Fig. 19/ Fig. 15	234	40
30	Fig. 13	Fig. 20	171	7
	* In millipolarization units			
	** In millipolarization units at a benzodiazepine concentration of 200 ng/ml Nordiazepam and a 5 ul sample size			

Sodium iodide (NaI) may be added to the sample or to one or more of the assay reagents other than the tracer in order to quench any fluorescence inherent in the sample, thus eliminating potential fluorescence interference in the assay. The amount of NaI employed is not critical, provided that a sufficient quantity is used to quench fluorescence by about fifty percent in the sample.

Additionally, largely conventional solutions including a pretreatment solution, a dilution buffer, benzodiazepines calibrator and benzodiazepines controls are desirably prepared. Typical solutions of these reagents, some of which are described below are commercially available in assay "kits" from Abbott Laboratories, Abbott Park, Illinois.

All percentages expressed herein are in weight/volume unless otherwise indicated. The tracer formulation presently preferred is 120 to 240 nanomolar tracer in 0.1 molar phosphate buffer at pH 7.5; 0.2% Polysorbate 80; 0.1% sodium azide; and 0.01% bovine gamma-globulin. The antisera formulation comprises sheep serum diluted with 0.1 molar tris buffer at pH 7.5; 0.1% sodium azide; 0.01% bovine gamma-globulin; and 2.0% ethylene glycol (volume/volume). The dilution buffer comprises 0.1 molar sodium phosphate at pH 7.5; 0.1% sodium azide; and 0.01% bovine gamma-globulin. The pretreatment solution comprises 50% sodium iodide; 0.9% sodium chloride; and distilled water. Benzodiazepine calibrators comprising nordiazepam in normal human urine at concentrations of 0.0, 200.0, 400.0, 800.0, 1200.0, and 2400.0 nanograms per milliliter with 0.1% sodium azide as a preservative are useful. Benzodiazepine controls comprised of nordiazepam in normal human urine at concentrations of 300.0 and 1000.0 nanograms per milliliter with 0.1% sodium azide as a preservative are also useful.

The fluorescence polarization value of each calibrator, control or samples is determined and is printed on the output tape of an instrument such as the Abbott TDx® polarization analyzer. A standard curve is generated in the instrument by correlating the polarization of each calibrator versus its concentration using a nonlinear regression analysis. The concentration of each control or sample is read off the stored calibration

curve and printed on the output tape. With respect to the foregoing preferred procedure, it should be noted that the tracer, antibody, pretreatment solution, calibrators and controls should be stored between about 2 and about 8 degrees C, while the dilution buffer should be stored at ambient temperature. A standard curve and controls should be run every two weeks, with each calibrator and control run in duplicate. Controls should be run daily and all samples can be run in replicates if so desired.

It should be understood that the foregoing detailed description and the following Examples are intended to be illustrative, but not limiting, with respect to the scope of the present invention. Various modifications will become apparent to one skilled in the art, and thus it is intended that the scope of the invention be defined solely by the claims and legal equivalents thereof.

EXAMPLES

Examples I through XXVII describe experiments that were performed in accordance with the concepts of the present invention. Example I and II are directed to preparations of immunogens useful for producing antibodies; Examples III through XIV are directed to the synthesis of precursors for immunogens and tracers; and Examples XV through XXVII are directed to the preparation of tracers.

Example I

Preparation of the Immunogen of Fig. 5

1-Carboxymethyl-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (56 mg) was combined with dicyclohexylcarbodiimide (60 mg) and N-hydroxy succinimide (32 mg) and pyridine (1 ml). The mixture was stirred for three hours. The mixture was added dropwise to 10 ml of a solution comprising of bovine serum albumin (10 mg/ml) in dimethylformamide (25%). This solution was then stirred at room temperature for 18 hours, after which it was filtered through glass wool to remove precipitate from reaction. The mixture was dialyzed in a cellulose dialysing tube (Spectra/Por 3, M.W. cutoff 3500) against 25% dimethylformamide for two days followed by dialysis against distilled water for three days. The solution from the dialysis tubing was found to contain 8.6 mg/ml protein via the Biuret protein concentration determining method.

Example II

Preparation of the Immunogen of Figure 13

50 mg (0.19 mmol) of 1-methyl-7-amino-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one was dissolved in 5.0 ml of N-Dimethylformamide (DMF), 1.5 ml of water and 0.5 ml of 1M hydrochloric acid. The mixture was cooled to 4°C and 0.15 ml of 1M sodium nitrite (0.15 mmol) was added to the above mixture. The reaction mixture was stirred at 4°C for 30 minutes. 125 ul of 1M urea in water was added to the mixture to quench the reaction. The resulting solution was added dropwise to a solution of bovine serum albumin (500 mg) in 12.5 ml of sodium borate buffer (0.1M, pH 9.0) and was stirred for one hour at 4°C (pH was maintained at 9.0 with 1 N NaOH).

This reaction mixture was maintained for 16 hours at 4°C with mixing. The resulting solution was dialyzed, first against 4 liters of sodium bicarbonate (0.05M) with 3 changes of the buffer. This was followed by dialyzing against 4 liters of deionized water (four changes). The resulting solution was the immunogen that would be injected into experimental animals.

Example III

1-[tert-Butoxycarbonylmethyl]-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 21)

To a solution of 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (264 mg, 9.7 mmol) in 20 ml of anhydrous N,N-dimethylformamide (DMF) under nitrogen was added sodium methoxide (659 mg, 12.8 mmol). The mixture was heated in an oil bath at 85°C for 30 minutes with stirring, and then a solution of tert-butyl bromoacetate (1.9 g, 9.7 mmol) in 1 ml of DMF was added over 10 minutes. After stirring was continued at the same temperature for 4 hrs, the mixture was concentrated in vacuo and partitioned between 90 ml of methylene chloride and 180 ml of water. The aqueous layer was extracted with two more portions of methylene chloride and the combined organic layers were washed with 45 ml of brine and dried (MgSO₄). After rotary evaporation the crude product was further dried in vacuo and purified by recrystallization from hexane/ethyl acetate (1:1) to give 1.99 g (53%) of a white powder.

Example IV1-Carboxymethyl-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 8)

To a solution of 1-[tert-butoxycarbonylmethyl]-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (275 mg, 0.71 mmol) in 5 ml of methylene chloride was added 2 ml of trifluoroacetic acid. After stirring at room temperature for 4 hours, the volatiles were removed in vacuo, and the residue was flash-chromatographed over silica gel (EM 9385). Elution with hexane/ethyl acetate (1:5) gave 240 mg of a light yellow glass.

Example V1-[(2-Aminoethylamine)carbonyl]methyl-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 10)

A mixture of 1-(carboxymethyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (35 mmol), dicyclohexylcarbodiimide (41 mg, 0.2 mmol), N-hydroxysuccinimide (13 mg, 0.11 mmol), and pyridine (0.25 ml) was stirred at room temperature for one hour. After the addition of ethylenediamine (30 mg, 0.5 mmol), the mixture was stirred for 5 additional hours. Water was then added and the aqueous mixture was extracted with ethyl acetate (three times). The combined extracts were washed with brine and dried over magnesium sulfate. Rotary evaporation of the solution gave a crude product which was recrystallized from ethyl acetate to give a colorless powder (24 mg) which had no aromatic proton in the NMR spectrum. The mother liquor was applied onto a preparative TLC plate (20 cm × 230 cm × 0.5 mm). Development with methanol-ammonium hydroxide (99:1) yielded 6.4 mg of ninhydrin positive (also UV active) material which gave the correct NMR spectrum.

General Procedure for Preparing DTAF Tracers (GI)

A mixture of the amine (0.01 mmol), DTAF(I or II) (0.01 mmol), triethylamine (2 drops) and methanol (0.1 ml) was stirred at room temperature for 16 hours. The mixture was applied onto a preparative silica gel TLC plate. Development with CHCl₃/MeOH (3:1 or 4:1) gave fluorescent bands which were scraped off the plate and eluted with methanol separately. In selected cases, the relatively pure tracer was further purified on a reverse-phase preparative TLC plate (Whatman 4803-800, KC-18 F254) using acetonitrile/0.01M phosphate buffer (pH 5.3), (1:1, v/v) as developer.

General Procedure for Preparing Carboxyfluorescein Tracers (GII)

A mixture of the amine (0.01 mmol), fluorescein carboxylic acid (V or VI)-O-succinimide ester (0.01 mmol) and pyridine (0.1 ml) was stirred at room temperature for 16 hours. The mixture was applied to a preparative TLC plate. Development with CHCl₃/MeOH (3:1 or 4:1) gave fluorescent bands which were scraped off the plate and eluted with methanol separately.

General Procedure for Preparing Fluoresceinamine Tracers (GIII)

A mixture of the carboxylic acid (0.01 mmol), dicyclohexylcarbodiimide (0.02 mmol) and *N*-hydroxysuccinimide (0.012 mol) in dry pyridine (0.1 ml) was stirred at room temperature for 1 hour. The active ester formed was then treated with fluoresceinamine (isomer I or II) at the same temperature for 16 hours. The reaction mixture was applied to a preparative silica gel tlc plate (20 cm x 20 cm x 0.5 mm). Development with CHCl₃/MeOH (3:1 or 4:1 depending on the polarity of the substrate) gave fluorescent bands which were scraped off the plate. The individual bands were eluted with methanol and the eluents were collected.

Example VI1-(2-Azidoethyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 22)

To a solution of 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (270 mg, 1 mmol) in 3 ml of anhydrous *N,N*-dimethylformamide (DMF) under nitrogen was added sodium methoxide (freshly prepared from 30 mg of sodium and 3 ml of methanol). The mixture was heated in an oil bath at 85°C for 15 minutes with stirring and then a solution of 2-azidoethyl mesylate in DMF (260 mg) was added. After stirring was continued for 8 hours, the mixture was concentrated and partitioned between water and methylene chloride (three times). The combined organic phases were washed with H₂O and brine (once each). After drying over magnesium sulfate, the solution was rotary evaporated to give a crude product (335 mg).

Example VII1-Aminoethyl-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 9)

A mixture of the crude azide (310 mg) prepared in Example VI, propane dithiol (1.1 ml), and triethylamine (0.9 ml) in 30 ml of methanol was stirred at room temperature overnight. The mixture was diluted with water and extracted with ethyl acetate (three times). The extracts were combined, washed with brine, and dried (magnesium sulfate). Rotary evaporation of the solution gave a crude residue which was flash-chromatographed over silica gel (120 ml). Elution with methanol-NH₄OH (700:1) gave a ninhydrin positive and UV active material (109 mg) which was characterized by high-field (360 MHz) proton NMR spectrum.

Example VIII1-(3-Azidopropyl)-7-chloro-5-phenyl-2H-1, 4-benzodiazepin-2-one

Solvent was removed from 0.375 ml of 1.0 M potassium ethoxide in ethanol. Dimethylformamide (1.0 ml) and 7-chloro-1,3-dihydro-5-phenyl-2H-1, 4-benzodiazepin-2-one (81 mg) were added, and the mixture was heated in an oil bath at 80°C for 15 minutes. After cooling to room temperature, 81 mg of 3-azidopropyl methanesulfonate in 0.15 ml of dimethylformamide was added and the mixture was again heated in the 80°C oil bath for one hour, when it had solidified to a gel. Partitioning between dichloromethane and water, washing with brine, drying with magnesium sulfate, filtration and removal of solvents gave the crude product, which was purified by chromatography on a thick layer silica gel plate developed with hexane-ethyl acetate to give the title compound.

Example IX

1-(3-Aminopropyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

A solution of 40 mg of 1-(3-azidopropyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one and 35 mg of triphenylphosphine in 2 1/2 ml of anhydrous THF was stirred at room temperature for 24 hours. Water (0.0026 ml) was added and stirring was continued for 3 hours more. Removal of volatile materials left a mixture of the title compound and triphenylphosphine oxide, which was used to prepare tracers without further purification.

10 Example X7-Chloro-1-(fluorescein-11-ylmethylaminocarbonylmethyl)-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (Fig. 19)

40 Milligrams of the acid prepared as described in Example IV was taken up in 2 ml of N,N-dimethylformamide and N-hydroxysuccinimide (15 mg) was added followed by 1,3-dicyclohexylcarbodiimide (28 mg). Stirring was continued at room temperature for 16 hours. This was filtered into a solution of aminomethylfluorescein (48 mg) and triethylamine (24 mg) in 3 ml of N,N-dimethylformamide. The mixture was stirred at room temperature for 24 hours.

PurificationFIRST -

Whatman C18, 20 × 20 cm./mm prep plates solvent system: 70/30/5 methanol, water, acetic acid $R_f = 0.2$

30 SECOND

Silica gel, 20 × 20 cm./mm prep plate solvent system: 90/10 methylene chloride-methanol $R_f = 0.65$

35 Example XI3-[(t-Butoxycarbonyl)methyl]-7-chloro-,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 23)

Prepared from diazepam by a procedure similar to that described in Example III.

Example XII3-(carboxymethyl)-7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 11)

For the procedure of removing the tert-butoxy group see Example IV.

Example XIIIF-Chloro-1,3-dihydro-1-methyl-5-phenyl-3-[(2-trimethylsilylethoxy)carbonylaminoethyl]-2H-1,4-benzodiazepin-2-one (Fig. 24)

A mixture of the carboxylic acid (prepared in Example XII, 85 mg, 0.248 mmol), diphenylphosphoryl azide (68 mg, 0.248 mmol), triethylamine (25 mg, 0.248 mmol) and toluene (1.5 ml) was stirred at 80°C for 2h under nitrogen. 2-(Trimethylsilyl)ethanol (59 mg, 0.499 mmol) was added and the resulting mixture was heated at the same temperature for additional 6 hours with stirring.

After cooling to room temperature, the mixture was partitioned between water and ethyl acetate. The combined organic layers were washed with brine (once) and dried over magnesium sulfate. Rotary-evaporation of the solution gave 171 mg of oil. One third of the sample was applied to a preparative TLC plate (silica gel, 20 cm x 20 cm x 2 mm). Development with ethyl acetate/hexane (5:1) gave a major band (R_f = 0.52) which was scraped off the plate. Eluting with methanol gave 26 mg of an oil.

Example XIV

10 3-Aminomethyl-7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 12)

To a solution of the carbamate (prepared according to Example XIII, 24 mg, 0.052 mmol) in THF (1 ml) was added via a syringe 0.21 ml of 1M (n-Bu)₄NF in THF. The resulting solution was stirred at 50°C for 30 minutes. Ethyl acetate was added and the diluted mixture was washed with ammonium chloride solution and water (once each). After drying over magnesium sulfate, the solution was rotary evaporated to afford 25 mg of an oil.

Example XV

20 1-2-[4-chloro-2-(fluorescein-5-ylamino)-1,3,5-triazin-6-ylamino]ethyl -7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one.

Prepared according to the general procedure (G1).

Example XVI

30 1-2-[4-chloro-2-(fluorescein-6-ylamino)-1,3,5-triazin-6-ylamino]ethyl -7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 15)

Prepared according to the general procedure (G1).

Example XVII

35 1-[2-(fluorescein-5-ylcarbonyl)aminoethyl]-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 14)

Prepared according to the general procedure (GII).

Example XVIII

45 1-[2-(fluorescein-6-ylcarbonylamino)ethyl]-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

Prepared according to the general procedure (GII).

Example XIX

50 1-[(fluorescein-5-ylamino)carbonyl methyl]-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

Prepared according to the general procedure (GIII).

Example XX

1-(fluorescein-6-ylaminocarbonylmethyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one.

Prepared according to the general procedure (GIII).

5

Example XXI

1-2-[4-chloro-2-(fluorescein-5-ylamino)-1,3,5-triazin-6-ylamino]ethyl amino carbonylmethyl -7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

10

Prepared according to the general procedure (GI).

Example XXII

15

1-2-[4-Chloro-2-(fluorescein-6-ylamino)-1,3,5-triazin-6-ylamino]ethyl aminocarbonyl methyl -7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 16)

Prepared according to the general procedure (GI).

20

Example XXIII

25

1-[2-(Fluorescein-5-ylcarbonyl amino)ethylaminocarbonylmethyl]-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

Prepared according to the general procedure (GIII).

30

Example XXIV

1-[2-(Fluorescein-6-ylcarbonylamino)ethylamino-carbonylmethyl]-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one

35

Prepared according to the general procedure (GII).

Example XXV

40

1-3-(2-[Fluorescein-6-ylamino]-4-chloro-1,3,5-triazin-2-ylamino)propyl -7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 17)

45

A solution of 11 micromoles of 1-(3-aminopropyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one and 5.8 mg of 2-(fluorescein-6-ylamino)-4,6-dichloro-1,3,5-triazine in 0.2 ml of methanol was stirred at room temperature for 15 minutes. One equivalent (0.0015 ml) of triethylamine was added, and stirring was continued for 27 hours. The crude product was twice chromatographed on silica gel thin layer plates, once with chloroform-methanol and once with benzene-ethyl acetate-acetone, to give the pure conjugate.

50

Example XXVI

55

1-(3-[Fluorescein-6-ylcarbonylamino]propyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 18)

A solution of 11 micromoles of 1-(3-aminopropyl)-7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one and 5.2 mg of 6-(N-succinimidylcarbonyl) fluorescein in 0.15 ml of dimethylformamide was stirred at room temperature for 4 1/2 hours. Solvent was removed *in vacuo* and the crude product was purified by chromatographing twice on thin layer silica gel plates, once with chloroform-methanol and once with benzene-ethyl acetate-acetone, to give the pure conjugate.

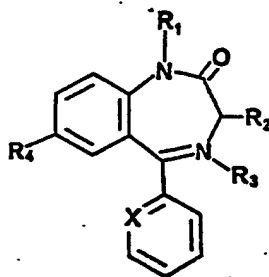
Example XXVII

1-Methyl-7-[2 (fluorescein-5-ylamino)-4-chloro-1,3,5-triazin-6-ylamino]-1-methyl-7-amino-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one (Fig. 20)

A mixture of 13.3 mg of 7-amino-1-methyl-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one, 0.021 ml of triethylamine and 26.5 mg of 2-(fluorescein-5-ylamino)-4,6-dichloro-1,3,5-triazine was stirred at ambient temperature overnight in 0.5 ml of methanol. The crude reaction mixture was streaked onto a silica gel thick layer plate and chromatographed with chloroform-methanol to give the pure conjugate.

Claims

1. A compound comprising the structure:



wherein:

X is CH, N or C-halogen;

R₁ is -H, -CH₃ or -R-Z-Q;

R₂ is -H or -OH;

R₃ is -O or a nonbonding electron pair;

R₄ is -R-Z-Q when neither R₁ is -H or -CH₃; or halogen, -NO₂, -NH₂ or -NHCOCH₃ when R₁ is -R-Z-Q;

R is a linking group consisting of from 0 to 20 carbon atoms and heteroatoms, including not more than twelve heteroatoms, arranged in a straight or branched chain and containing up to two ring structures, with the proviso that not more than four heteroatoms may be linked in sequence, nor may more than two sulfur or nitrogen or one oxygen atom be linked in sequence;

Z is C=O, C=NH, NH, NCH₃, -N=N-, SO₂ or CH₂; and

Q is hydrogen, hydroxyl, halogen, acyloxy, N-succinimidyl, N-phthalimidyl, alkoxy, phenoxy, substituted phenoxy, N-imidazolyl, 1-benzotriazolyl, or is a poly(amino acid), poly(amino acid) derivative or another immunogenic carrier substance, or is an amino, amido, anidino, urea, thiourea, carbamate, thiocarbamate, triazinylamino, or (carboxyamino)-sulfonamido derivative of fluorescein.

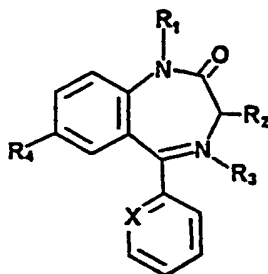
2. The compound of Claim 1 wherein Q is bovine serum albumin.

3. The compound of Claim 1 wherein Q is a triazinylamino derivative of fluorescein.

4. The compound of Claim 1 wherein Q is 4-chloro-6-(fluorescein-6-ylamino)-1,3,5-triazin-2-yl, fluorescein-5-yl carbonyl, or fluorescein-6-yl carbonyl.

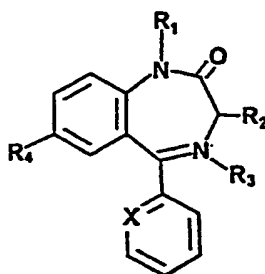
5. The compound of Claim 1 wherein Q is (fluorescein-5-ylamino)carbonyl or (fluorescein-6-ylamino)carbonyl.

6. The compound of Claim 1 wherein Q is (fluorescein-5-yl)amino or (fluorescein-6-yl)amino.
 7. An antibody to a compound according to Claim 1 wherein Q is a non-enzymatic poly(amino acid) or a non-enzymatic poly(amino acid) derivative or another immunologically active carrier.
 8. A method for making an immunogen comprising the step of coupling a precursor of the formula:



wherein:

- X is CH, N or C-halogen;
 R₁ is -H, -CH₃ or -R-Z-Q;
 R₂ is -H or -OH;
 R₃ is -O or a nonbonding electron pair;
 R₄ is -R-Z-Q when neither R₁ is -H or -CH₃, or halogen, -NO₂, -NH₂ or -NHCOCH₃ when R₁ is -R-Z-Q;
 R is a linking group consisting of from 0 to 20 carbon atoms and heteroatoms, including not more than twelve heteroatoms, arranged in a straight or branched chain and containing up to two ring structures, with the proviso that not more than four heteroatoms may be linked in sequence, nor may more than two sulfur or nitrogen or one oxygen atom be linked in sequence;
 Z is C=O, C=NH, SO₂, NH, NCH₃ or CH₂; and
 Q is hydrogen, hydroxyl or a leaving group (with the proviso that when Z is CH₂, Q may not be hydrogen) with a poly(amino acid), a poly(amino acid) derivative or another immunogenic carrier.
 9. The method of Claim 8 wherein when ZQ is NH₂ or CO₂H, the precursor is coupled by reacting it with the poly(amino acid) by addition of N,N'-dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide or 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate.
 10. The method of Claim 8 wherein when ZQ is NHCH₃ or NH₂ and the precursor is coupled by reacting it with the poly(amino acid) by addition of 1-ethyl-2-(3-dimethylaminopropyl) carbodiimide.
 11. The method of Claim 8 wherein when ZQ is NHCH₃, NH₂ or CH₂OH and when X₁ is N, the precursor is coupled by the steps of:
 (a) reacting the resulting compound from step (a) with phosgene or thiophosgene;
 (b) reacting the product of step (a) with the poly(amino acid)
 12. A method for making a tracer comprising the step of coupling a precursor of the formula;



wherein:

- X is CH, N or C-halogen;
 R₁ is -H, -methyl or -R-Z-Q;
 R₂ is -R-Z-Q when neither R₁ nor R₄ is -R-Z-Q or it is -H or -OH;
 R₃ is -O or a nonbonding electron pair;
 R₄ is -R-Z-Q when neither R₁ nor R₂ is -R-Z-Q, or it is halogen, -NO₂, -NH₂ or -NHCOCH₃;

R is a linking group consisting of from 0 to 20 carbon atoms and heteroatoms, including not more than twelve heteroatoms, arranged in a straight or branched chain and containing up to two ring structures, with the proviso that not more than four heteroatoms may be linked in sequence, nor may more than two sulfur or nitrogen or one oxygen atom be linked in sequence;

5 Z is NH, CO, SO₂ or C=NH; and

Q is -H, -OH, a leaving group with fluorescein or a derivative of fluorescein.

13. The method of Claim 12 wherein when ZQ is NH₂, the precursor is coupled by the steps of:

(a) preparing an active ester of carboxyfluorescein;

(b) reacting the active ester with the precursor.

10 14. The method of Claim 12 wherein when ZQ is CO₂H, the precursor is coupled by the steps of:

(a) preparing an active ester of the precursor;

(b) reacting the active ester with aminofluorescein.

15 15. The method of Claim 12 wherein ZQ is NH₂ and the precursor is reacted with (4,6-dichloro-1,3,5-triazin-2-ylamino)fluorescein or fluorescein isothiocyanate.

16. The method of Claim 12 wherein a precursor is coupled by the steps of:

(a) preparing an imidate ester of the precursor; and

(b) reacting the imidate with aminofluorescein.

17. The method of Claim 12 wherein when Q is OH, the precursor is coupled by reacting it with (4,6-dichloro-1,3,5-triazin-2-ylamino)fluorescein.

20 18. A process for detecting the presence of benzodiazepines and benzodiazepine metabolites which comprises the steps of:

(a) contacting a sample with a benzodiazepine derivative antiserum and with a compound according to Claim 1 capable of producing a detectable fluorescence polarization response to the presence of the benzodiazepines derivative antiserum;

25 (b) passing plane polarized light through the resulting solution from step (a) to obtain a fluorescence polarization response; and

(c) detecting the fluorescence polarization response of the solution of step (b) as a measure of the presence of benzodiazepines and benzodiazepine metabolites in the sample.

30 19. The process of Claim 18 wherein the benzodiazepines derivative antiserum is produced by the antibodies of Claim 7.

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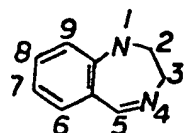


FIG. 1

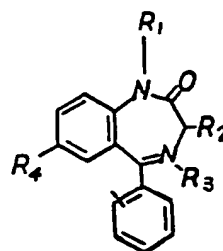


FIG. 2

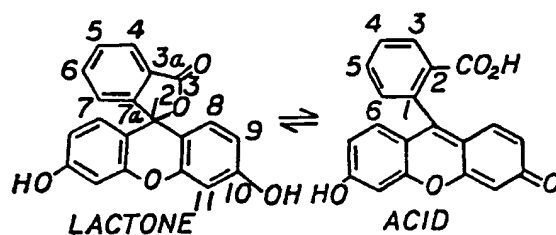


FIG. 3

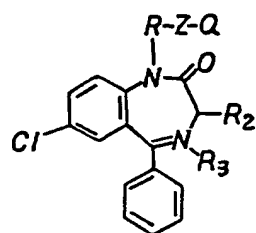


FIG. 4

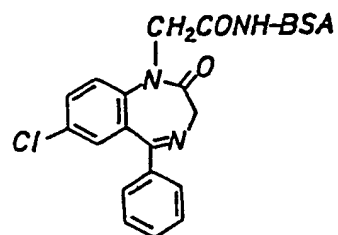


FIG. 5

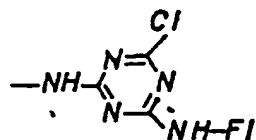


FIG. 7-1

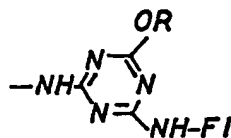


FIG 7-2

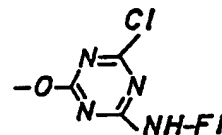


FIG 7-3



FIG. 7-4



FIG. 7-5

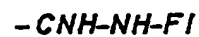


FIG. 7-6



FIG. 7-7



FIG. 7-8



FIG. 7-9



FIG. 7-10

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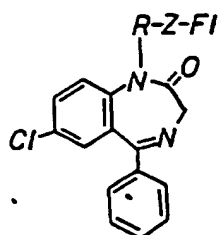


FIG. 6

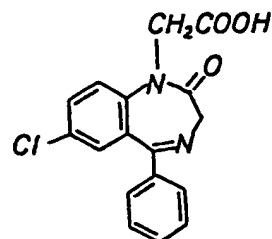


FIG. 8

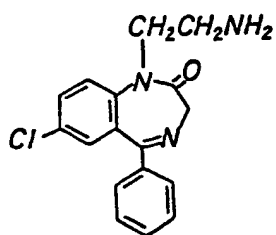


FIG. 9

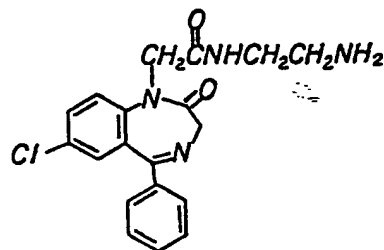


FIG. 10

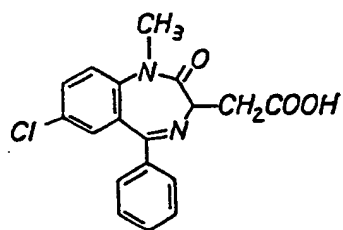


FIG. 11

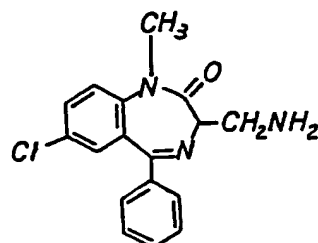
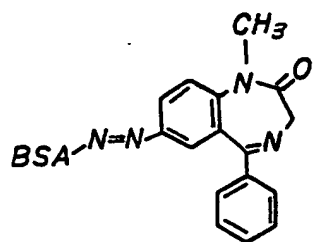
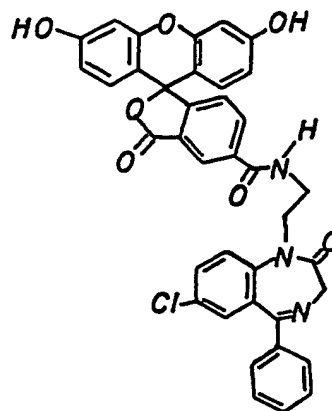
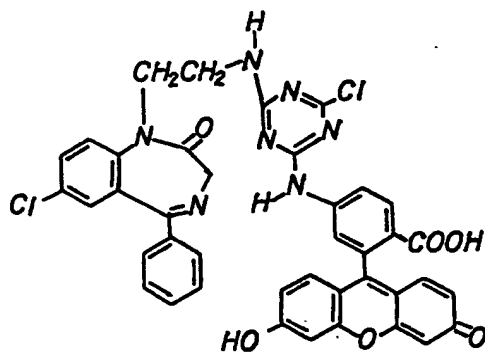
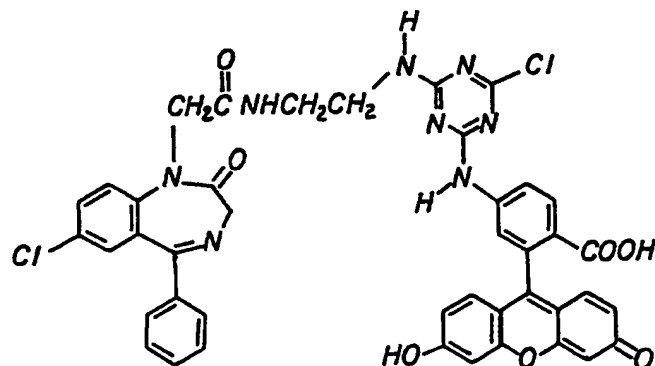


FIG. 12

FIG. 13FIG. 14FIG. 15FIG. 16

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Nouvellement déposé

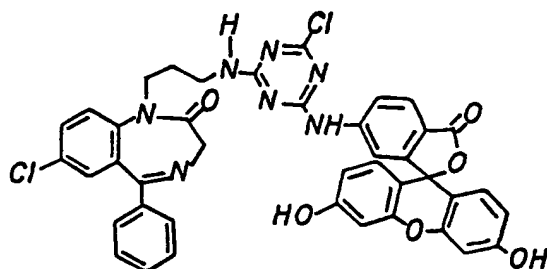


FIG. 17

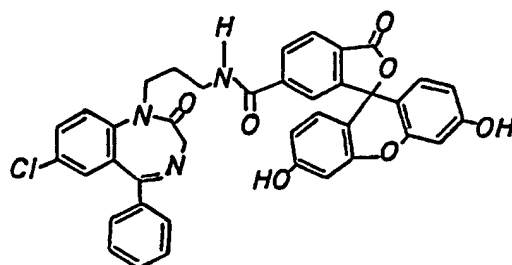


FIG. 18

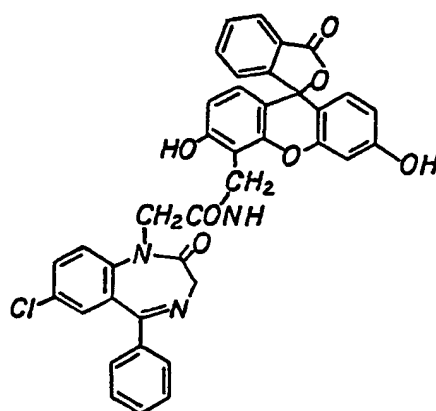


FIG. 19

Not valid for priority
Not valid for priority

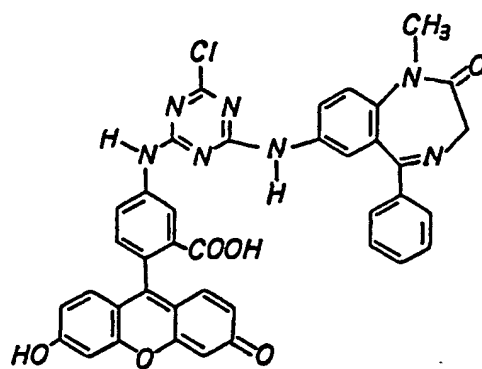


FIG. 20

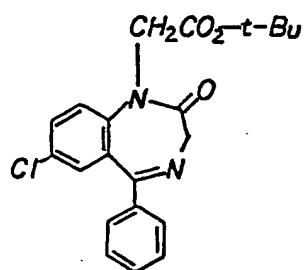


FIG. 21

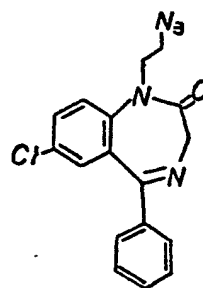


FIG. 22

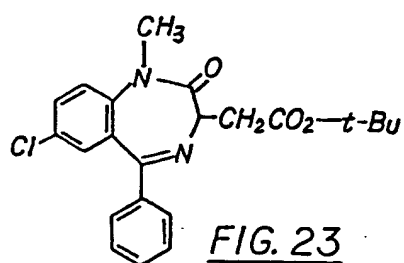


FIG. 23

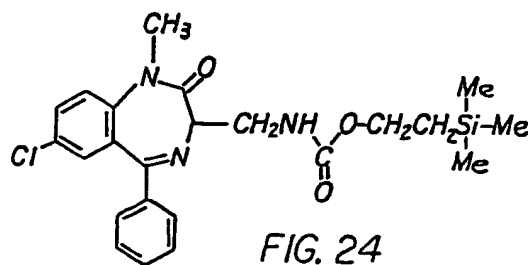


FIG. 24